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## EUROPEAN PATENT SPECIFICATION

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⑤④ **Amylose-lysozyme hybrid, an activated sugar and processes for producing them.**

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## Description

This invention relates to a hybrid molecule formed by a sugar having a reducing terminal and containing no carboxyl group linked to lysozyme through a peptide bridge and a process for producing the same, and the object is to enhance the stability of lysozyme. Further, the other object is to provide an activated sugar comprising a sugar having a reducing terminal and containing no carboxyl group linked to N-hydroxysuccinimide through a peptide bridge, which activated sugar is necessary to produce the above sugar-lysozyme hybrid, and a process for producing the activated sugar.

Proteins are characterized by a primary structure formed by linking 20 kinds of amino acids and a steric structure defined by the above structure. It has already been known that proteins have various functions, and even proteins stable in vivo become unstable when used in vitro.

When superior functions of proteins are applied to various uses, proteins have the following drawbacks:

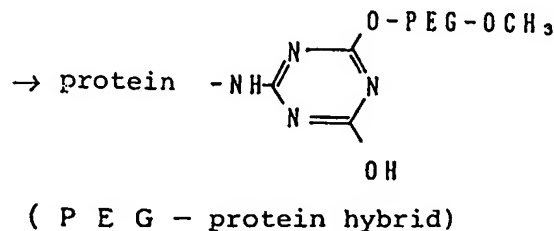
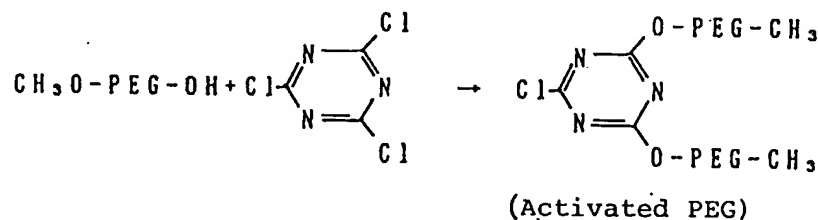
- ① they are unstable to heat, alkalies and acids and liable to be denatured;
- ② they are insoluble in organic solvents and liable to lose their activity;
- ③ they have antigenic properties; etc.

In order to overcome these drawbacks, proteins have been chemically modified. Such chemical modification of proteins into a Protein hybrid has made it possible to compensate the above drawbacks. Various processes have been proposed for such chemical modification. Among these processes, the most often employed one is a process of using as a modifying agent, a polyethylene glycol (hereinafter abbreviated to PEG) which is a non-immunity synthetic high-molecular weight compound.

According to the above process, as illustrated below in the equations, a synthetic compound (an activated PEG) is prepared from monomethoxypolyethylene glycol and a cyanuric chloride (2,4,6-trichloro-S-triazine), followed by reacting this activated PEG with a protein to produce a PEG-protein hybrid.

Utilizing this protein-hybrid, various application examples as shown below have been reported, but practically commercially employed examples appear to be few.

This is because the activated PEG is unstable; no product having uniform properties is obtained; furthermore cyanuric chloride is poisonous and the reaction of the activated PEG with proteins does not advance quantitatively and smoothly.



The application examples of PEG-protein hybrid are shown below.

(1) PEG-asparaginase (T. Pharmac., Y. Kamisaki et al; Exp. Therap. 216, 410).

It prolongs the half-life period in blood, of asparaginase as an antitumor enzyme and reduces its antigenic properties.

(2) By utilizing a PEG-enzyme hybrid, enzyme reactions have been made possible even in organic solvents (Y. Imada et al; Trends in Biotechnology 4 190(1986), K. Takahashi et al; J. Org. Chem 50 3414-

(1985) K.Takahashi et al;Enzyme 32 235(1984). K.Takahashi et al;Biochem Biophys Res. Commun:125 761(1984)).

Catalase, lipase, chymotrypsin, peroxidase, etc.

(3) PEG-adenosine deaminase (M.S. Hershfield et al; N. Engl. J. Mol. 316,493 (1985))

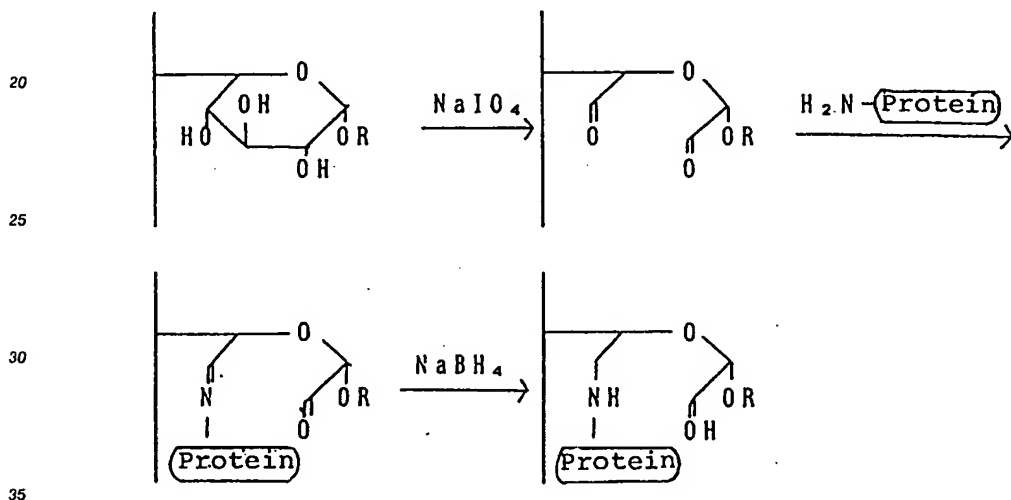
5 Among genetic enzyme-deficient phenomena, there is adenosine aminase (ADA)-deficient phenomenon. When this ADA is administered, if it is made up into PEG-ADA hybrid, it has been reported that this hybrid has effects of notably prolonging the half-life period in blood, etc.

(4) PEG-interleukin 2 (Taiji Imoto; Chemistry and Organism, Vol. 27, page 426, 1989)

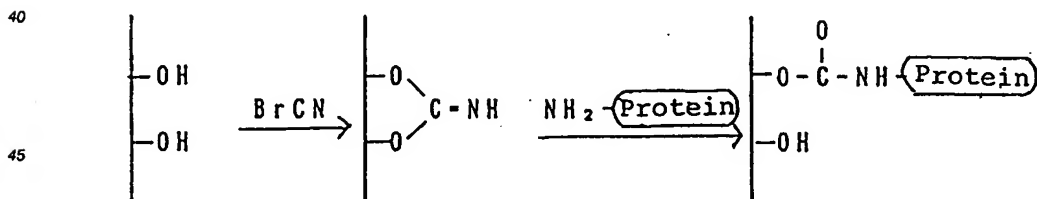
10 Interleukin 2 which is a kind of lymphokines has been mass-produced according to recombinant DNA technique, but it is deficient in sugar chain so that it is unstable, but when it is made up into PEG-interleukin 2 hybrid, it could have been stabilized and also its antitumor effect could have been improved.

In order to make up a hybrid with proteins, sugars have been also utilized besides PEG. As processes utilizing sugars, there are the following processes (i) to (iv), and the effects of the resulting hybrids are almost the same as those in the case of PEG:

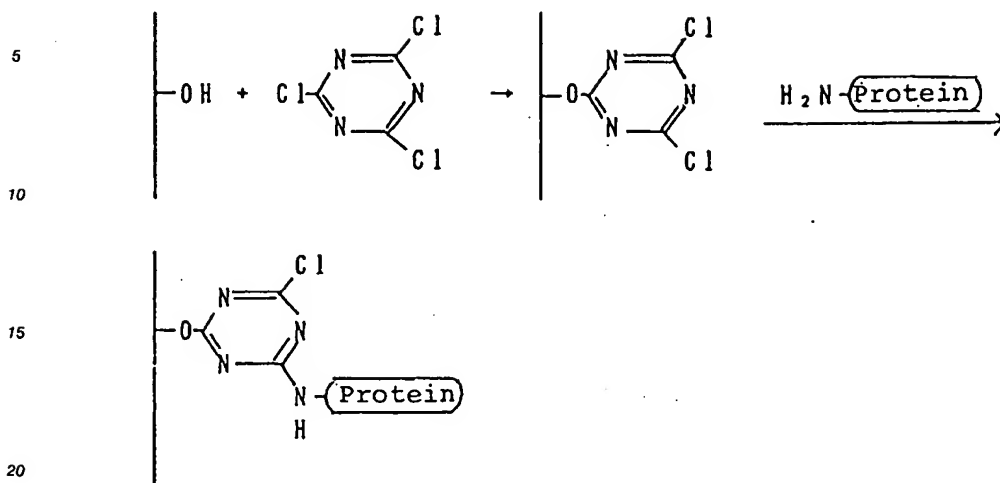
15 (i) Periodic acid oxidation process:



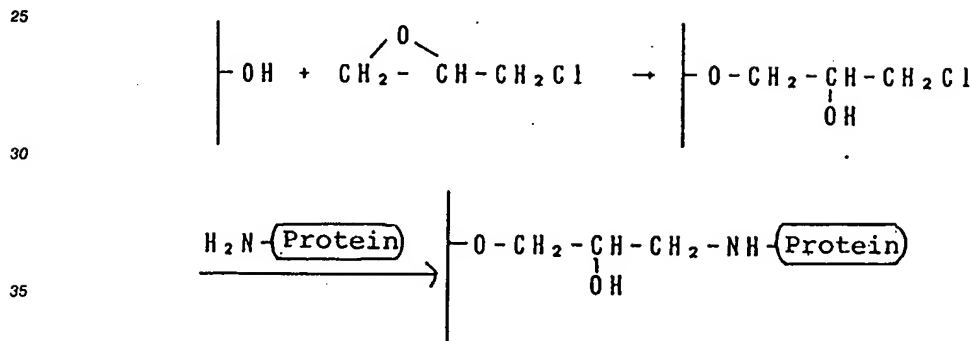
(ii) Bromine cyanide process



## (iii) Cyanuric chloride process



## (iv) Epichlorohydrin process



The process (i) has drawbacks that the reaction with periodic acid is so severe that sugars are often decomposed; binding with proteins requires to use a reducing agent; there is a possibility that proteins are denatured; etc.

The process (ii) has drawbacks that poisonous bromine cyanide is used; when proteins are bound, it is necessary to severely adjust the pH in the process; etc.

The process (iii) has drawbacks that cyanuric chloride is poisonous; the reaction of cyanuric acid with sugars does not smoothly advance; etc.

The process (iv) has drawbacks that sugars crosslink with each other due to epichlorohydrin; etc. The drawbacks common to these processes consist in that since the above respective substances react with -OH of the constituting sugars, the binding position is indefinite and also since they react with any -OH of sugars, the properties specific of the respective sugars are lost.

Lysozyme as a bacteriolytic enzyme has been broadly used for medical industry, etc., and in order to enhance its stability, high-level techniques are required or cumbersome operations are required; for example, the composition of constituting amino acids are varied according to gene engineering technique; crosslinking is carried out between the objective amino acids (see, Taiji Imoto; Chemistry and Organism, Vol. 27, page 426, 1989).

The present invention has the following three main objects:

(1) An object is to provide an activated sugar useful for reacting a sugar having a reducing terminal and containing no carboxyl group with the amino group of a protein only at the reducing terminal of the sugar, without using any poisonous reagent, to thereby smoothly effect the reaction of combining the

sugar with the protein, without losing the intrinsic properties of the sugar.

(2) Another object is to provide a hybrid of lysozyme with a sugar, utilizing the above activated sugar to thereby enhance the stability of lysozyme.

(3) A still further object is to provide processes for producing the above activated sugar and the above sugar-lysozyme.

Other objects will be apparent from the description mentioned below.

The present invention relates to:

(1) A hybrid molecule formed by a sugar having a reducing terminal and containing no carboxyl group linked to lysozyme through a peptide bridge.

(2) A hybrid according to item (1), wherein said sugar is amylose.

(3) A hybrid according to item (1), wherein said peptide is glycylglycine.

(4) A hybrid according to item (1), wherein said sugar is at least one member selected from the group consisting of monosaccharides, oligosaccharides and polysaccharides.

(5) An activated sugar comprising a sugar having a reducing terminal and containing no carboxyl group linked to N-hydroxysuccinimide, through a peptide bridge.

(6) An activated sugar according to item (5), wherein said peptide is glycylglycine.

(7) An activated sugar according to item (5), wherein said sugar is at least one member selected from the group consisting of monosaccharides, oligosaccharides and polysaccharides.

(8) A process for producing an activated sugar, which comprises reacting a sugar having a reducing terminal and containing no carboxyl group, with a peptide comprising an amino group at one end and a carboxyl group at the other end in the presence of a reducing agent, followed by reacting N-hydroxysuccinimide with the resulting material in the presence of a condensation agent.

(9) A process for producing an activated sugar according to item (8), wherein said peptide is glycylglycine.

(10) A process for producing an activated sugar according to item (8), wherein said sugar is at least one member selected from the group of monosaccharides, oligosaccharides and polysaccharides.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figs. 1 to 4 each show an explanatory chart illustrating Examples of the present invention.

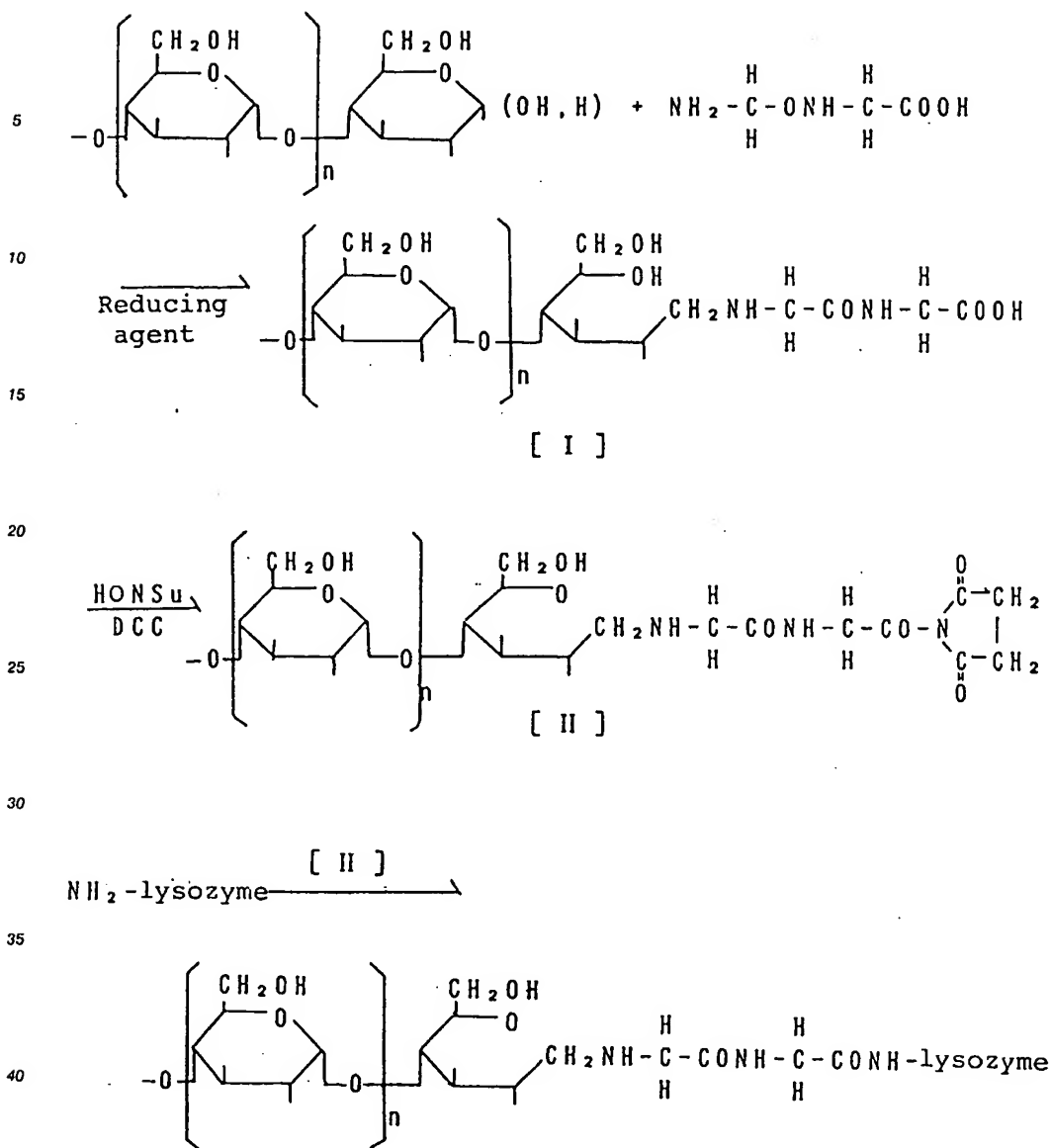
#### DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

##### A. Preparation of activated sugar:

##### (1) First step:

A sugar having a reducing terminal is dissolved in a buffer solution or an organic solvent such as dimethylsulfoxide, etc., followed by adding to the solution, a peptide having an amino group at one end and a carboxyl group at the other end and a reducing agent and reacting the mixture to prepare a compound as expressed by the formula (I) mentioned below.

The buffer solution in this case has no particular limitation, but it may contain no amino group and have a pH of 5 to 9. The sugar is a monosaccharide, a oligosaccharide or a polysaccharide. The peptide has no particular limitation, but the number of the constituting amino acids is suitably 2 to 10. As the reducing agent, sodium borohydride (hereinafter abbreviated to SBH), sodium cyanoborohydride ( $\text{NaBH}_3\text{CN}$ , hereinafter abbreviated to SCBH) and dimethylaminoborane ( $(\text{CH}_3)_2\text{HNBH}_3$ , hereinafter abbreviated to DMAB) are preferred. The reaction temperature is preferably 10° to 60°C. After completion of the reaction, unreacted peptide and reducing agent are separated and removed by means of gel filtration or ultrafiltration membrane.



## (2) Second step:

The above compound of the formula (I) is dissolved in a buffer solution or an organic solvent, followed by adding to the solution, N-hydroxysuccinimide (hereinafter abbreviated to HONSu) and a condensation agent, to prepare a compound of the formula (II). The condensation agent is suitably dicyclohexylcarbodiimide (hereinafter abbreviated to DCC), 1-ethoxycarbonyl-2-ethoxy-1,2-dihydroxyquinoline (hereinafter abbreviated to EEDQ), disuccinimide carbonate (hereinafter abbreviated to DSC), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (hereinafter abbreviated to EDC), etc.

The compound of the formula (II) is a compound having a peptide sugar combined with HONSu and a novel compound so far not obtained and in a form readily reactive with the amino group of a protein (hereinafter referred to as activated sugar).

## B. Preparation of lysozyme-sugar hybrid:

The activated sugar [II] is reacted with lysozyme in a buffer solution containing no amino group, followed by separating a sugar-lysozyme hybrid from the activated sugar [II] by means of gel filtration chromatography or ultrafiltration membrane.

The above reaction advances almost quantitatively, but in the case where unreacted lysozyme is present, it is possible to obtain a lysozyme-sugar hybridoma by separating lysozyme from the sugar-lysozyme hybrid by means of CM-ion exchanger as a cation exchanger.

As described above, according to the present invention, it is possible to obtain a lysozyme bound to the reducing terminal of the sugar-sugar hybrid in a comparatively simple manner. This is epoch-making. With proteins other than lysozyme, too, it is possible to prepare a sugar-protein hybrid.

## C. Stability of sugar-lysozyme hybrid:

In order to observe to what extent the stability of the sugar-lysozyme hybrid has been enhanced as compared with lysozyme alone, the activity of lysozyme against heat was examined. The activity of lysozyme was measured using glycol chitin as a substrate.

As a result, the activity of unmodified lysozyme notably lowered at 80°C or higher, whereas sugar-lysozyme hybrid could retain its activity as far as 80% even at high temperatures of 90°C to 100°C; hence such a high stability is epoch-making.

As the sugar, not only amylose but also aminopectine, chitosan, dextran, agarose, etc. are employed.

Sugars having carboxyl group are undesirable to apply to the present process since they cause intermolecular crosslinkings of proteins with one another.

When pharmaceuticals of enzyme proteins are administered in the form of themselves, the half-life period is so short that they are rapidly excreted from kidney or decomposed so that the drug efficacy often cannot be expected. The present invention is applied to such increase in the stability of enzyme proteins in blood, increase in the retention time thereof in blood, etc. In an Example described later, there will be described an increase in the staying time in blood, of superoxide dimustase (hereinafter abbreviated to SOD) which is an enzyme specifically decomposing the superoxide anion of active enzymes and having recently come to be used for remedy of blood vessel obstacles in brain and heart, Behcet's disease, etc.

(Example)

The present invention will be described in more detail by way of Examples, but it should not be construed to be limited thereto.

Example 1

## Preparation of amylose-glycylglycine

Amylose (weight average molecular weight: 29,000) (1.0 g) was dissolved in a 0.1 M phosphoric acid buffer (pH: 8.5) (10 ml), followed by adding glycylglycine in a quantity by mol of 5 times that of amylose and SCBH in a quantity by mol of 50 times that of amylose, agitating the mixture at 80°C for 2 days, adjusting the pH to 3 with conc. hydrochloric acid, further agitating at 60°C for 5 hours and adjusting the pH to 7 with N-NaOH.

The resulting reaction liquid is subjected to gel chromatography with a gel-filtering medium (Trademark: Cellulofine GCL-25) to remove unreacted glycylglycine SCBH. The gel filtration results are shown in Fig. 1. The first peak portion is separated. The subsequent peak is of glycylglycine SCBH. The separated liquid was freeze-dried. In addition, the gel filtration was carried out under conditions of column: 1.2 x 60 cm, eluent: water, and flow rate: 10 ml/hr. 0.9 g of amylose-glycylglycine was obtained.

Example 2

## Preparation of activated amylose:

Amylose-glycylglycine (0.5 g) obtained in Example 1 was dissolved in dimethylsulfoxide (2 ml), followed by adding HONSu and DCC, each in a quantity by mol of 10 times that of amylose-glycylglycine, and agitating the mixture at room temperature overnight.

The resulting insolubles were filtered, followed by adding acetone (20 ml), agitating the mixture at 3,000 r.p.m. for 5 minutes, filtering off deposited precipitates and drying under reduced pressure to obtain activated amylose (0.4 g).

### 5 Example 3

Preparation of amylose-lysozyme hybrid

Lysozyme (11 mg) was dissolved in a 0.1 M boric acid buffer (pH: 8.5), followed by adding the  
10 activated amylose (17.2 mg) obtained in Example 2 and agitating the mixture at room temperature overnight.

The resulting material was dialyzed with 0.1 M phosphoric acid buffer (pH: 6.0) + 0.1 M NaCl, followed by filtering off insolubles, and subjecting the resulting filtrate to gel chromatography with a gel-filtering medium (trademark: Cellulofine GCL-300). The results are shown in Fig. 2. The former peak portion was  
15 separated. The, subsequent peak portion was of unreacted activated amylose. In addition, gel filtration was carried out under conditions of column: 1.5 x 64 cm, buffer: 0.1 M phosphoric acid buffer + 0.1% NaCl, and flow rate: 10.2 ml/hr.

The separated portion was desalted, followed by freeze-drying to obtain amylose-lysozyme hybrid (8  
20 mg).

### Example 4

Measurement of lysozyme activity

Lysozyme or amylose-lysozyme hybrid (0.1 ml) was added to a 0.1% glycol chitin solution (1 ml),  
25 followed by allowing the mixture to stand at 40 °C for 30 minutes, adding 0.05%  $K_3Fe(CN)_6$  (2 ml), boiling the mixture for 15 minutes and measuring an absorption at 420 nm. The relationship between the protein concentration and the absorbance is shown in Fig. 3.

### 30 Example 5

Stability of amylose-lysozyme hybrid:

A lysozyme solution or an amylose-lysozyme hybrid solution (500  $\mu$ l) was allowed to stand still at  
35 20 °C, 80 °C, 90 °C and 100 °C for 30 minutes.

Further, it was allowed to stand at room temperature for 2.5 hours, followed by measuring its lysozyme activity according to the method of Example 4

The results are shown in Fig. 4. Amylose-lysozyme hybrid retained 90% of the activity of lysozyme. As  
40 apparent from Fig. 4, at the time of treatment at 100 °C for 30 minutes, the activity of the unmodified lysozyme notably lowered, whereas the activity of amylose-lysozyme hybrid was retained at 90%, that is, the heat stability was far enhanced.

### Example 6

45 Preparation of dextran-glycylglycine:

Glycylglycine (0.5 g) was dissolved in water (60 ml), followed by adding triethylamine (7 ml) and dextran (average molecular weight 10,000) (1 g) and SCBH (0.3 g) dissolved in water (40 ml), agitating the mixture at 38 °C for 4 days and subjecting the resulting reaction solution to gel filtration in the same manner  
50 as in Example 1 to obtain dextran-glycylglycine (0.85 g).

### Example 7

Preparation of activated dextran:

55 Dextran-glycylglycine (0.5 g) obtained in Example 6 was dissolved in dimethylsulfoxide (5 ml), followed by adding HONSu (0.1 g) and DCC (0.15 g), agitating the mixture at room temperature overnight, filtering off insolubles, adding acetone (30 ml), agitating the mixture at 3,000 r.p.m. for 5 minutes, filtering



deposited precipitates and drying under reduced pressure to obtain an activated dextran (0.35 g).

#### Example 8

##### 5 Preparation of dextran-SOD hybrid:

SOD originated from *Bacillus stearothermophilus* (purchased from Biochemical Industry Co., Ltd.) (10 mg) (ca. 100,000 U) was dissolved in a 0.1 M phosphoric acid buffer (pH: 7.5) (10 ml), followed by adding the activated dextran (30 mg) obtained in Example 7 and agitating the mixture at room temperature  
10 overnight.

The resulting material was dialyzed with 0.1 M phosphoric acid buffer (pH: 6.0) + 0.1M NaCl, followed by filtering off insolubles, subjecting the resulting filtrate to gel chromatography in the same manner as in Example 3, desalting the separated portion and freeze-drying to obtain dextran-SOD hybrid (7mg).

##### 15 Example 9

Test of dextran-SOD hybrid remaining in blood:

SOD or dextran-SOD hybrid prepared in Example 8, each in 1,200 U, were intravenously injected into  
20 respective mice. In the case of administration of unmodified SOD, the SOD activity level in blood after 30 minutes was nearly zero, whereas in the case of the mouse having dextran-SOD hybrid administered, the activity level was retained at 90%.

#### Claims

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1. A hybrid molecule formed by a sugar having a reducing terminal and containing no carboxyl group linked to lysozyme through a peptide bridge.

2. A hybrid according to claim 1, wherein said sugar is amylose.

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3. A hybrid according to claim 1, wherein said peptide is glycylglycine.

4. A hybrid according to claim 1, wherein said sugar is at least one member selected from the group consisting of monosaccharides, oligosaccharides and polysaccharides.

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5. An activated sugar comprising a sugar having a reducing terminal and containing no carboxyl group linked to N-hydroxysuccinimide, through a peptide bridge.

6. An activated sugar according to claim 5, wherein said peptide is glycylglycine.

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7. An activated sugar according to claim 5, wherein said sugar is at least one member selected from the group consisting of monosaccharides, oligosaccharides and polysaccharides.

8. A process for producing an activated sugar, which comprises reacting a sugar having a reducing terminal and containing no carboxyl group, with a peptide comprising an amino group at one end and a carboxyl group at the other end in the presence of a reducing agent, followed by reacting N-hydroxysuccinimide with the resulting material in the presence of a condensation agent.

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9. A process for producing an activated sugar according to claim 8, wherein said peptide is glycylglycine.

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10. A process for producing an activated sugar according to claim 8, wherein said sugar is at least one member selected from the group of monosaccharides, oligosaccharides and polysaccharides.

#### Patentansprüche

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1. Hybridmolekül, gebildet durch einen Zucker mit einem reduzierenden Ende und ohne Carboxylgruppe, der an Lysozym über eine Peptidbrücke gebunden ist.

2. Hybrid nach Anspruch 1, dadurch **gekennzeichnet**, daß der Zucker Amylose ist.
3. Hybrid nach Anspruch 1, dadurch **gekennzeichnet**, daß das Peptid Glycylglycin ist.
- 5 4. Hybrid nach Anspruch 1, dadurch **gekennzeichnet**, daß der Zucker mindestens eine Verbindung, ausgewählt aus der Gruppe bestehend aus Monosacchariden, Oligosacchariden und Polysacchariden, ist.
- 10 5. Aktivierter Zucker, der einen Zucker mit einem reduzierenden Ende und ohne Carboxylgruppe aufweist, der an N-Hydroxysuccinimid über eine Peptidbrücke gebunden ist.
6. Aktivierter Zucker nach Anspruch 5, dadurch **gekennzeichnet**, daß das Peptid Glycylglycin ist.
- 15 7. Aktivierter Zucker nach Anspruch 5, dadurch **gekennzeichnet**, daß der Zucker mindestens eine Verbindung, ausgewählt aus der Gruppe bestehend aus Monosacchariden, Oligosacchariden und Polysacchariden, ist.
- 20 8. Verfahren zur Herstellung eines aktivierten Zuckers, dadurch **gekennzeichnet**, daß ein Zucker mit einem reduzierenden Ende und ohne Carboxylgruppe mit einem Peptid, das eine Aminogruppe am Ende und eine Carboxylgruppe am anderen Ende aufweist, in Anwesenheit eines Reduktionsmittels umgesetzt wird und anschließend N-Hydroxysuccinimid mit dem entstehenden Material in Anwesenheit eines Kondensationsmittels umgesetzt wird.
- 25 9. Verfahren zur Herstellung eines aktivierten Zuckers nach Anspruch 8, dadurch **gekennzeichnet**, daß das Peptid Glycylglycin ist.
- 30 10. Verfahren zur Herstellung eines aktivierten Zuckers nach Anspruch 8, dadurch **gekennzeichnet**, daß der Zucker mindestens eine Verbindung, ausgewählt aus der Gruppe der Monosaccharide, Oligosaccharide und Polysaccharide, ist.

#### Revendications

1. Molécule hybride formée d'un sucre ayant une extrémité réductrice et ne contenant pas de groupe carboxyle lié au lysozyme par un pont peptidique.
- 35 2. Hybride selon la revendication 1, dans lequel ledit sucre est l'amylose.
3. Hybride selon la revendication 1 dans lequel ledit peptide est la glycylglycine.
- 40 4. Hybride selon la revendication 1, dans lequel ledit sucre est au moins un membre choisi au sein du groupe consistant en monosaccharides, oligosaccharides, et polysaccharides.
5. Sucre activé comprenant un sucre ayant une extrémité réductrice et ne contenant pas de groupe carboxyle lié au N-hydroxysuccinimide par un pont peptidique.
- 45 6. Sucre activé selon la revendication 5, dans lequel ledit peptide est la glycylglycine.
7. Sucre activé selon la revendication 5, dans lequel ledit sucre est au moins un membre choisi au sein du groupe consistant en monosaccharides, oligosaccharides, et polysaccharides.
- 50 8. Procédé pour la production d'un sucre activé, qui comprend la réaction d'un sucre ayant une extrémité réductrice et ne contenant pas de groupe carboxyle, avec un peptide comprenant un groupe amino à une extrémité, et un groupe carboxyle à l'autre extrémité, en présence d'un agent réducteur, suivie de la réaction du N-hydroxysuccinimide avec le produit ainsi obtenu, en présence d'un agent de condensation.
- 55 9. Procédé pour la production d'un sucre activé selon la revendication 8, dans lequel ledit peptide est la glycylglycine.

10. Procédé pour la production d'un sucre activé selon la revendication 8, dans lequel ledit sucre est au moins un membre choisi au sein du groupe des monosaccharides, oligosaccharides, et polysaccharides.

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FIG. 1

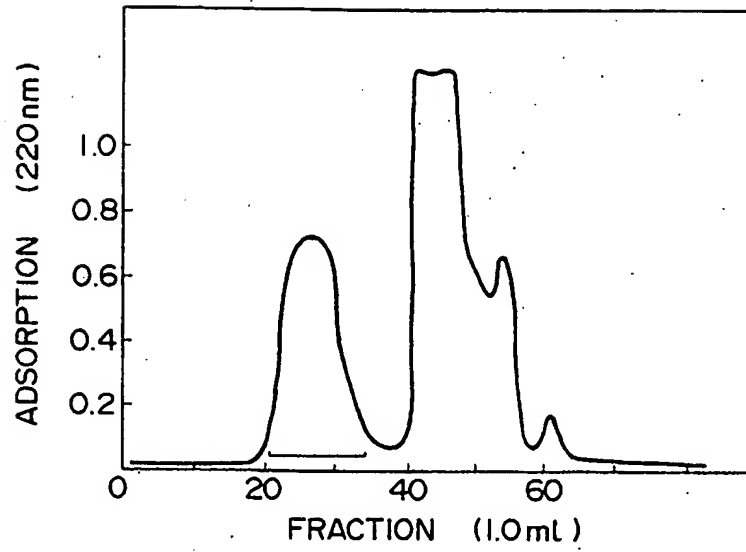


FIG. 2

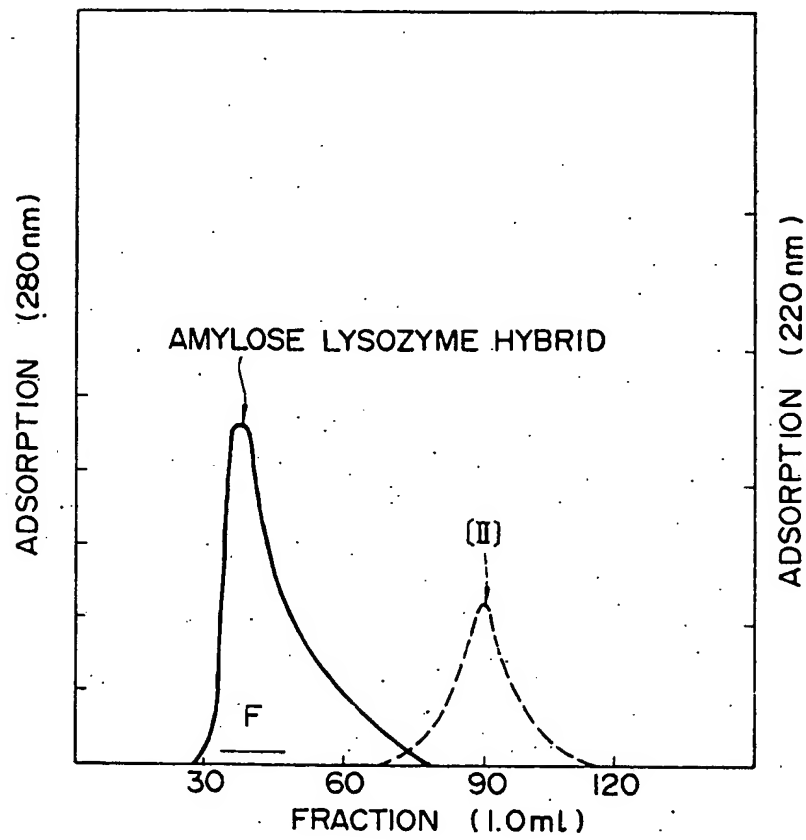


FIG. 3

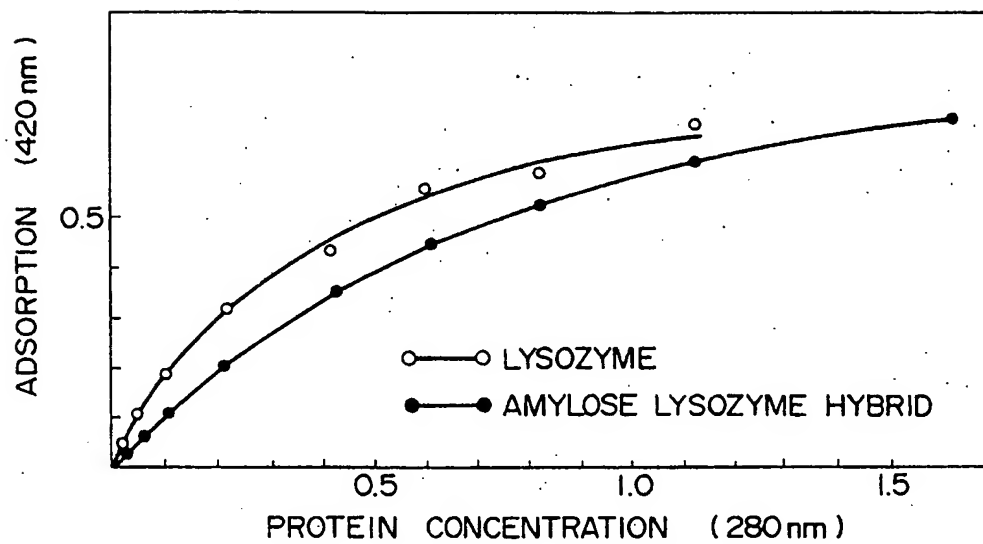


FIG. 4

